

# Immunohistochemical analysis of development of desmin-positive hepatic stellate cells in mouse liver

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## ABSTRACT

Development of desmin-positive hepatic stellate cells was studied in mice using double immunofluorescent techniques and *in vitro* cultures with special attention given to their cell lineages. Several studies recently reported on the presence of cells that are immunologically reactive with both antidesmin and anticytokeratin antibodies in young fetal rat livers, and suggested the possibility that these cells give rise to hepatocytes and hepatic stellate cells. At early stages of mouse liver development, stellate cells with desmin-positive filaments were scattered in the liver parenchyma. However, the stellate cells definitely differed from hepatoblasts and hepatocytes in terms of their morphology and expression of desmin and hepatoblast and hepatocyte-specific E-cadherin in the liver. Fetal hepatoblasts and hepatocytes did not react with antidesmin antibodies, nor did desmin-positive stellate cells express E-cadherin *in vivo* and *in vitro*. Thus it is likely that desmin-positive stellate cells and hepatoblasts belong to different cell lineages. In the fetal liver, the desmin-positive stellate cells surrounded blood vessels, and extended their processes to haematopoietic cells and megakaryocytes. Many, but not all, hepatoblasts and hepatocytes were observed to be associated with the stellate cells. At fetal stages, cellular processes positive for desmin in the stellate cells were also thick compared with those in the adult liver, in which desmin-positive stellate cells lay in Disse's space and were closely associated with all hepatocytes. These developmental changes in the geography of desmin-positive cells in the liver parenchyma and their morphology may be associated with their maturation and interactions with other cell types.

*Key words:* Cell lineage; hepatoblasts; E-cadherin; cytokeratins; double immunostaining.

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## INTRODUCTION

The mammalian fetal liver, which is a haematopoietic organ, is comprised of hepatoblasts and various types of nonparenchymal cells, including haematopoietic cells, precursors of hepatic stellate cells (Ito cells) and endothelial cells (Enzan et al. 1997; Godlewski et al. 1997). Hepatoblasts produce and secrete  $\alpha$ -fetoprotein (AFP) (Shiojiri, 1981; Shiojiri et al. 1991) and albumin (Cascio & Zaret, 1991; Shiojiri et al. 1991), but do not express any other markers of mature hepatocytes such as carbamoylphosphate synthase I (CPSI), a urea cycle enzyme (Dingemans et al. 1996). These cells, which originate from endodermal cells of the hepatic primordium, give rise to mature hepatocytes and biliary epithelial cells during liver development

(Shiojiri, 1984, 1994; Van Eyken et al. 1988). Hepatic stellate cells, which settle in the perisinusoidal space of Disse and express desmin, an intermediate filament protein (Yokoi et al. 1984; Tsutsumi et al. 1987), are known to have important functions. These include storage of vitamin A (Blomhoff et al. 1985) and production of the extracellular matrix (ECM) in adult liver (Friedman et al. 1985; Milani et al. 1989). When activated, these cells transform into potent fibre-producing cells and play a pivotal role in liver fibrosis (Gressner & Bachem, 1995; Gressner, 1998; Ikeda et al. 1998; Mallat et al. 1998). However, not only their roles but also their origin are still unclear in fetal liver development.

Recently, some groups reported that at early stages of rat liver development, most nonhaematopoietic

cells, including hepatoblasts, had transient co-expression of desmin and epithelial cell-specific cytokeratins in liver (Vassy et al. 1993; Kiassov et al. 1995). Based on these results, they proposed the idea that nonhaematopoietic desmin-positive cells give rise to both hepatocytes and hepatic stellate cells. On the other hand, it has also been considered that hepatic stellate cells are derived from the septum transversum mesenchyme (Enzan et al. 1983, 1997).

However, the expression of cytokeratins is not specific for hepatoblasts and hepatocytes in the fetal rodent liver. Cytokeratins are also expressed in mesothelial cells and biliary epithelial cells (Van Eyken et al. 1988; Shiojiri et al. 1991; Shiojiri, 1994). Markers that only hepatoblasts express but non-parenchymal cells do not express should be used to demonstrate the lineages of hepatoblasts. E-cadherin, one of the epithelial cell adhesion molecules, may be an ideal marker for hepatoblasts at early stages of liver development (Ogou et al. 1983; Takeichi, 1988).

In the present study, we analysed the expression of desmin in hepatoblasts in mouse liver development using double immunofluorescent techniques for desmin and E-cadherin, or cytokeratins to investigate whether the hypothesis on the desmin-positive cell origin of hepatoblasts and hepatic stellate cells is true in the fetal mouse liver. Desmin expression in fetal mouse liver cells cultured *in vitro* was studied to confirm our results *in vivo*. We further examined developmental changes of the distribution pattern of desmin-positive cells in liver parenchyma to find clues to the understanding of roles that hepatic stellate cells play in liver development.

## MATERIALS AND METHODS

### *Materials*

C3H/HeSlc mice (SLC, Hamamatsu, Japan) were used. The animals were mated during the night. If a copulative plug was present the next morning, this day was considered as 0.5 d of gestation. Fetuses at 9.5, 11.5, 12.5, 13.5, 14.5, 15.5 and 17.5 d of gestation, newborns (males, 1 d old), and 1-wk-old, 2-wk-old and 12-wk-old (adult) animals (males) were used in the present study. At least 3 animals at each stage, and 6 sections of each liver were examined for immunostaining.

### *Immunohistochemistry*

For immunofluorescence of desmin, cytokeratins and E-cadherin, whole young fetuses or liver tissues were

frozen in n-hexane chilled by dry ice-ethanol. Frozen sections (8 µm thickness) were cut, and fixed in acetone for 7 min at -30 °C. Tissues for immunostaining of AFP, albumin and CPSI were fixed in a chilled mixture of 95% ethanol and acetic acid (99:1 v/v) overnight, and embedded in paraffin. Serial paraffin sections (6 µm thickness) were cut.

Rabbit antiserum against chicken desmin (Medac, Hamburg, Germany) (1/100 dilution with phosphate-buffered saline [PBS] containing 1% bovine serum albumin [BSA]), rat monoclonal antibodies against mouse E-cadherin (ECCD-2) (Takara Biomedicals, Otsu, Japan) (1/200 dilution with 20 mM tris[hydroxymethyl]aminomethane-HCl [pH 7.4]-buffered saline [TBS] containing 10 mM CaCl<sub>2</sub> and 1% BSA), guinea pig antiserum against bovine cytokeratins 8 and 18 (Progen Biotechnik, Heidelberg, Germany) (1/200 dilution), rabbit antiserum against mouse AFP (Organon Teknika, Durham, NC, USA) (1/100 dilution), rabbit antiserum against mouse albumin (Organon Teknika) (1/100 dilution) and rabbit antiserum against rat CPSI (a generous gift from Dr W. H. Lamers) (Lamers et al. 1984) (1/1000 dilution) were used as primary antibodies. Hydrated tissue sections and cultured fetal liver cells were incubated with the primary antibodies for 1 h at room temperature. After being thoroughly washed with PBS, the samples were incubated with fluorescein isothiocyanate (FITC)-labelled goat antibodies against rabbit immunoglobulin G (IgG) or rat IgG (Organon Teknika) (1/100 dilution), or FITC-labelled donkey antibodies against guinea pig IgG (Jackson Immuno-Research West Grove, PA, USA) (1/500 dilution) for 1 h, washed again, and mounted in buffered glycerol containing p-phenylenediamine (Johnson & de C Nogueira Araujo, 1981). The specific immunofluorescence in the samples was observed with a fluorescence microscope (model BHS-RF) (Olympus, Tokyo, Japan). In the case of E-cadherin immunostaining, TBS was used as the washing solution. The control slides were incubated with PBS-1% BSA or TBS-1% BSA in place of the primary antibodies. The control slides for desmin immunostaining were also incubated with the antiserum which had been absorbed with purified chicken desmin (90 µg/ml; Progen Biotechnik).

For double immunostaining (desmin-E-cadherin, desmin-cytokeratins, and E-cadherin-cytokeratins), the following species-specific secondary antibodies were used; 5-([4,6-dichlorotriazin-2-yl] amino)fluorescein-labelled donkey antibodies against rabbit IgG (1/400 dilution), indocarbocyanine-labelled donkey antibodies against rat IgG (1/500 dilution), indo-

carbocyanine-labelled donkey antibodies against guinea pig IgG (1/500 dilution), and FITC-labelled goat antibodies against rat IgG (1/100 dilution) (Jackson ImmunoResearch).

#### *Quantitation of the number of desmin-positive cells*

The number of desmin-positive cells was counted on the photographic prints of desmin immunofluorescence (0.11 mm<sup>2</sup>; 6 different areas for each animal). In 11.5 to 15.5 d fetal livers, counting was done in the whole liver parenchyma without distinguishing between periportal and pericentral areas. In 17.5 d, neonatal and adult livers, counting was carried out in periportal, pericentral and midzonal areas. The periportal or pericentral areas were within 0.03 mm from portal or central veins, respectively. The results were expressed as mean desmin-positive cell number per unit area for each stage. The areas were measured using a computer-assisted image analyser (Luzex-F, Nireco Corp., Hachioji, Japan).

#### *Immunoblotting*

Fresh adult liver tissues were homogenised at 4 °C in a lysis buffer (125 mmol/l Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS]), and were then solubilised by boiling for 5 min. Protein content was determined by using the BCA protein assay reagent kit (Pierce Chemical, Rockford, IL, USA).

SDS-polyacrylamide gel electrophoresis (PAGE) was performed on 10% acrylamide slab gels under reducing conditions following the method of Laemmli (1970). Each sample that was loaded onto the gel contained 20 µg of total protein. Following electrophoresis, proteins were transferred to nitrocellulose membranes. The membranes were blocked with Block Ace (Dai-Nippon Seiyaku, Osaka, Japan) prior to incubation with primary antibodies (rabbit antichick desmin antiserum [1/2000 dilution], guinea pig antibovine cytokeratins antiserum [1/2000 dilution], or rat antiE-cadherin antibodies [1/2000 dilution]) at room temperature overnight. After being washed with TBS containing 0.2% Tween-20, horseradish peroxidase-conjugated goat antibodies against rabbit, guinea pig or rat IgG (ICN ImmunoBiologicals, Lisle, IL, USA) were used at a 1/2000 dilution for 2 h at room temperature. ECL detection was conducted with Amersham Pharmacia Biotech (Buckinghamshire, England) reagents according to the manufacturer's recommendations. In control experiments, antichick desmin antiserum was absorbed with

purified chicken desmin (12 µg/ml) before incubation of blots.

#### *Primary culture*

Fetal livers at 12.5 d of gestation were dissected free of adhering tissue, diced, and incubated with dispase (1000 U/ml) (Godo Shusei, Tokyo, Japan) in 2-[4-(hydroxyethyl)-1-piperazinyl]ethanesulphonic acid (Sigma, St Louis, MO, USA)-buffered Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) for 30 min at 37 °C. The liver specimens were subsequently dispersed by vortexing (Bennett et al. 1987), and filtrated through a nylon mesh filter (pore size 132 µm) (Nihon Rikagaku Kikai Tokyo, Japan). After the filtrate was left for 30 min on ice, the haematopoietic cell-rich fraction was removed by aspiration using a pipette. The remaining cell suspension was centrifuged at 80 × g for 10 min. The cellular pellet was resuspended in DM-160 (Kyokuto Seiyaku, Tokyo, Japan) containing 10% fetal calf serum (FCS)(Gibco), penicillin G potassium (100 U/ml) (Meiji Seika, Tokyo, Japan), streptomycin sulphate (100 µg/ml) (Meiji Seika) and 0.01% deoxyribonuclease I (Worthington Biochemicals, Freehold, NJ, USA) (Lamers et al. 1984). The centrifugation step was repeated 4 times to remove dispase perfectly. Finally, the cellular pellet was resuspended in DM-160-10% FCS containing 10<sup>-7</sup> M dexamethasone (Yeoh et al. 1985; Molero et al. 1994; Roncero et al. 1995) and the antibiotics. The viability of the cells was more than 95% by the Trypan blue exclusion test. The fetal liver cells (10<sup>6</sup> cells/ml, 70 µl) were cultured on a glass area (a diameter of 6 mm) surrounded by a Teflon-coated area on glass slides (HT-coating-slide) (Ea Brown Ltd., Tokyo, Japan) at 37 °C in a water-saturated atmosphere containing 5% CO<sub>2</sub> for 24–72 h. Medium change was performed after 24 h of the start of culture. For the immunofluorescence, the cultured cells were fixed in cold acetone after being washed with PBS.

## RESULTS

### *Reactivity of antidesmin, anticytokeratins and antiE-cadherin antibodies*

Immunoblotting analysis demonstrated that antidesmin antiserum, anticytokeratins antiserum and antiE-cadherin antibodies specifically reacted with major bands of 53 kDa, 47 kDa and 55 kDa, and 110 kDa (data, not shown) in adult mouse liver

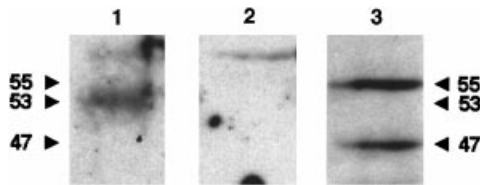


Fig. 1. Immunoblotting analysis of reactivity of antidesmin and anticytokeratin antisera in adult liver extract. Immunoblotting was carried out as described. Lanes 1, 2 and 3 show incubation of the blots with antidesmin antiserum, preabsorbed antidesmin antiserum and anticytokeratins antiserum, respectively. A 53 kDa band of desmin which is detected with antidesmin antiserum is not visible in incubation of the blot with the preabsorbed antiserum. The antiserum to bovine cytokeratins reacts with 2 major cytokeratin polypeptides of 47 kDa and 55 kDa in adult mouse liver.

extracts, respectively (Fig. 1). Preabsorption of antidesmin antiserum with purified chicken desmin abolished positive immunostaining of a 53 kDa desmin band.

#### *Distribution of desmin-positive cells during development of mouse liver*

In the 9.5-d liver primordium, the endodermal tissue was supported by the septum transversum mesenchyme (Fig. 2*b, c*). Most of the mesenchymal cells were desmin-positive, and they were spindle-shaped rather than stellate. The hepatic endodermal cells did not express desmin.

In the 11.5-d fetal liver parenchyma, stellate-shaped desmin-positive cells were scattered among foci of desmin-negative hepatoblasts and haematopoietic cells (Fig. 3*a*). They protruded their long and thin desmin-positive processes to the foci of haematopoietic cells, and contacted haematopoietic cells and megakaryocytes. Their desmin-positive processes were thicker than those of adult hepatic stellate cells (Fig. 3*b, 4d*). Hepatoblasts were also located close to them, but not all hepatoblasts were associated with them at this stage. Although we could observe desmin-positive cells discontinuously surrounding blood vessels with clear lumina, it was uncertain whether all desmin-positive cells were located underneath endothelial cells of blood vessels on fluorescence microscopy (Fig. 7*a, d*). Basically, this distribution pattern of the desmin-positive cells in the liver parenchyma did not change at later stages of fetal liver development, though from around 13.5 d of gestation onwards desmin-positive connective tissue developed around portal veins (Fig. 3*c, d*).

In neonatal livers, cellular processes containing desmin-positive filaments in stellate cells appeared to be finer than those of fetal stellate cells (Fig. 4*a*). However, the distribution pattern of these cells in

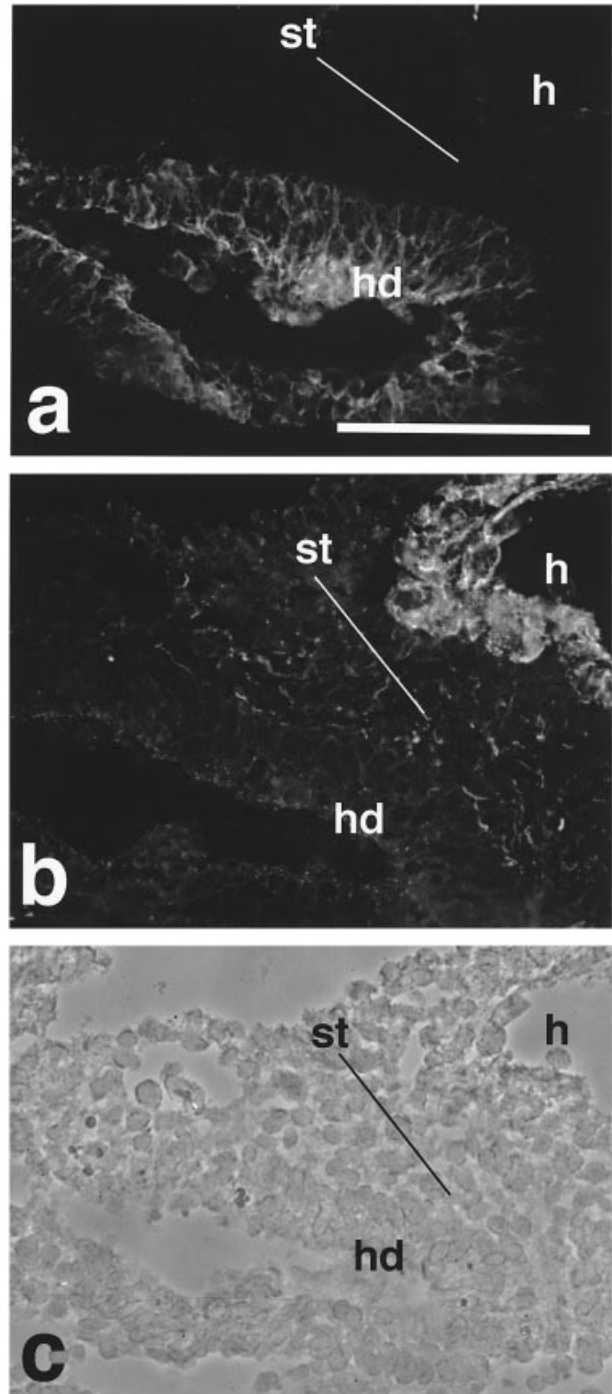


Fig. 2. Immunolocalisation of E-cadherin (*a*) and desmin (*b*) in 9.5 d liver primordium. (*a*) The lateral cell membrane near the apical surface of the endodermal cells of the hepatic diverticulum (hd) is strongly positive for E-cadherin. (*b*) The section adjacent to *a*. Desmin-positive filaments are found in most of mesenchymal cells of the septum transversum (st), which are spindle-shaped rather than stellate. (*c*) Phase-contrast micrograph of the section adjacent to *b*. h, heart. Bar (*a*), 100 µm.

the liver parenchyma, including their geographical relationships with haematopoietic cells, hepatocytes and blood vessels, was still basically the same as at fetal stages. With postnatal liver development, desmin-

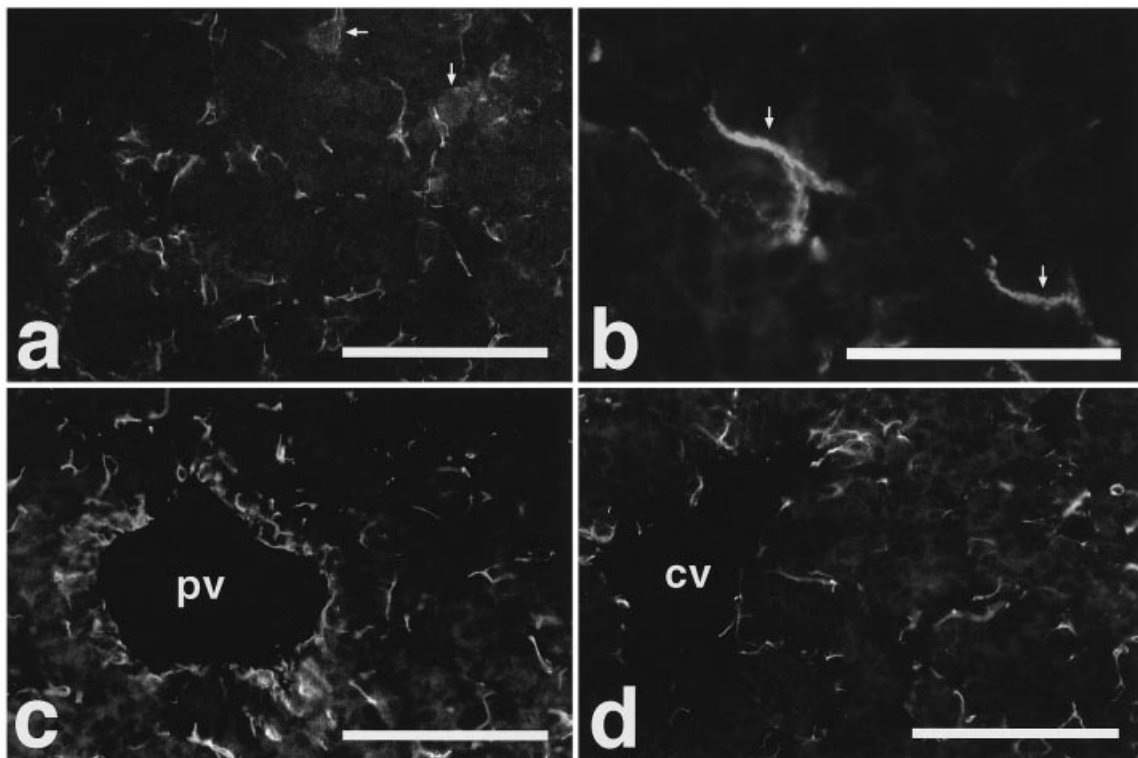


Fig. 3. Immunolocalisation of desmin in fetal mouse livers at 11.5 d (*a*), 14.5 d (*b*) and 17.5 d (*c, d*) of gestation. (*a*) Desmin-positive stellate cells protrude long cell processes containing desmin-positive filaments, and contact megakaryocytes (arrows). (*b*) A higher magnification of fetal stellate cells (arrows). (*c, d*) Desmin-positive cells are more frequently observed in periportal areas than in pericentral or midzonal areas. cv, central vein; pv, portal vein. Bars (*a, c, d*), 100  $\mu$ m; (*b*), 40  $\mu$ m.

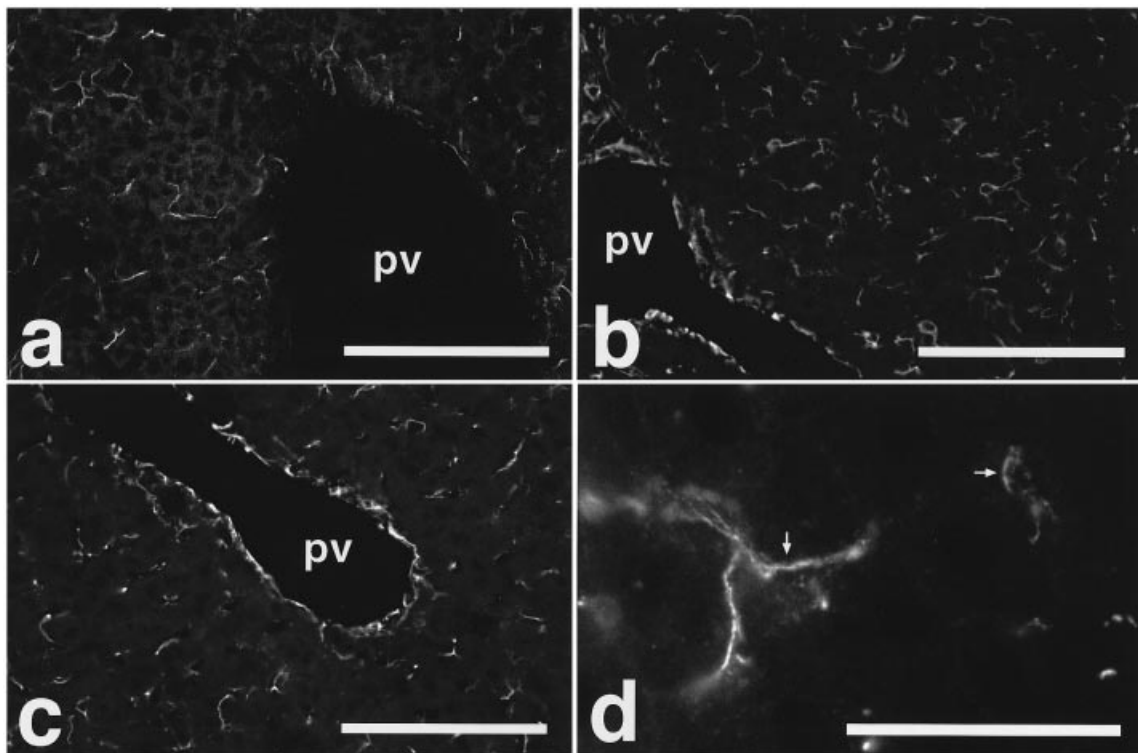


Fig. 4. Distribution of desmin-positive stellate cells in neonatal (*a*), 2-wk-old (*b*) and adult (*c, d*) mouse livers. (*a*) The width of desmin-positive cytoplasmic processes of stellate cells become shorter than those of the fetal cells. (*b*) Desmin-positive stellate cells distribute more uniformly in the liver parenchyma. (*c*) Stellate cells are associated with all hepatocytes and sinusoidal endothelial cells. A high density of desmin-positive cells is found in periportal connective tissue. (*d*) A higher magnification of adult stellate cells (arrows). pv, portal vein. Bars (*a, b, c*), 100  $\mu$ m; (*d*), 40  $\mu$ m.

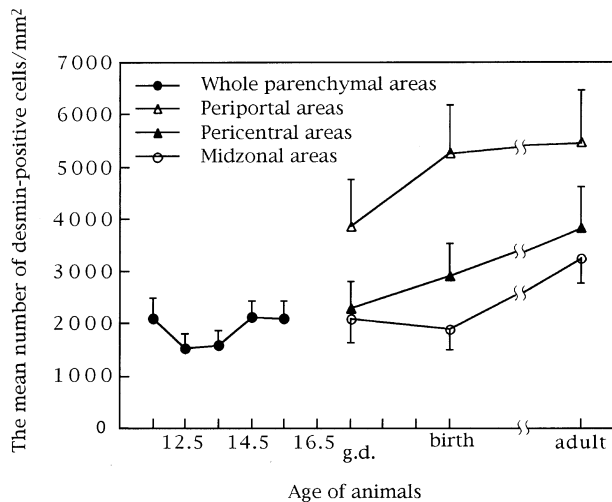


Fig. 5. Developmental changes of the density of desmin-positive cells in mouse livers. Each point represents the mean of the density of desmin-positive cells. The midzonal areas correspond to the parenchymal area between the periportal and pericentral areas. Bars indicate the standard deviations of the means. g.d., gestation days.

positive stellate cells were more uniformly distributed (Fig. 4*b, c*). In adult liver, stellate cells with finer desmin-positive processes were located between the hepatic cell plates and the sinusoidal endothelium (Fig. 4*c, d*), and all hepatocytes were closely associated with these stellate cells (1 or 2 stellate cells for each hepatocyte).

Control slides including incubation of tissue sections with absorbed antidesmin antiserum or PBS-BSA in place of the primary antibody were invariably negative.

The density of desmin-positive cells in the liver parenchyma also changed with the progress of development (Fig. 5). It somewhat decreased from 11.5 d to 12.5 d, and then increased from 13.5 d onwards. Separate analysis of the densities of desmin-positive cells in periportal, pericentral and the midzonal areas of 17.5 d, neonatal and adult liver parenchyma showed that the number of desmin-positive cells in the unit area was higher in the periportal areas than in other areas at all stages examined. Their values in the 3 areas reached a peak in adult liver (Fig. 5).

#### *Expression of AFP, albumin, E-cadherin, cytokeratins and CPSI in developing mouse liver*

In fetal livers, hepatoblasts and hepatocytes were strongly positive for both AFP and albumin immunostaining (Fig. 6*a, b*). Their shapes were cuboidal compared with those of desmin-positive cells (mostly

stellate), though hepatoblasts were smaller than hepatocytes. Positive immunoreactions of both serum proteins were also seen in endothelial cells, connective tissue cells and some blood cells.

At 9.5 d of gestation, E-cadherin was localised on the cell membrane of endodermal cells of the liver primordium (Fig. 2*a*). Mesenchymal cells of the septum transversum were negative for E-cadherin. E-cadherin was also expressed on the cell membrane of the endoderm-derived cells such as hepatoblasts, hepatocytes and biliary epithelial cells during later liver development. The E-cadherin immunostaining was highly specific for these cells, and clearly demonstrated that hepatoblasts were not stellate, but cuboidal (Fig. 6*c*). Periportal hepatocytes were more positively stained for E-cadherin than hepatocytes in other areas. Intrahepatic bile duct cells and their precursors, which developed around portal veins from 13.5 d of gestation onward, had higher levels of E-cadherin expression than hepatoblasts and hepatocytes (Fig. 6*e, f*). Other cell types, including stellate cells, endothelial cells, connective tissue cells, haematopoietic cells and mesothelial cells were negative for E-cadherin throughout fetal liver development.

Networks of cytokeratin-positive filaments were found in the cytoplasm of hepatoblasts, hepatocytes and mesothelial cells throughout liver development (Fig. 6*d*). Strong signals of cytokeratins were detected in intrahepatic bile duct cells and their precursors (Fig. 6*g, h*).

CPSI expression started in hepatocytes at 15.5 d of gestation. Although precursors of intrahepatic bile duct cells expressed CPSI weakly, the expression was highly specific to hepatocytes at later stages of liver development (data not shown).

#### *Double immunofluorescent analysis*

Double immunostaining for desmin and E-cadherin or cytokeratins was performed to examine whether hepatoblasts positive for E-cadherin and cytokeratins expressed desmin at 11.5 and 12.5 d of gestation. E-cadherin immunostaining was found in hepatoblasts, but was not detected in desmin-positive stellate cells (Fig. 7*a-c*). Most desmin-positive stellate cells never expressed cytokeratins which were seen in hepatoblasts and mesothelial cells at these stages (Fig. 7*d-f*), but a few of them (6% of total desmin-positive cells) had positivity for cytokeratin immunostaining. Co-expression of E-cadherin and cytokeratins was found in hepatoblasts. By contrast, cytokeratin-positive mesothelial cells were negative for E-cadherin.

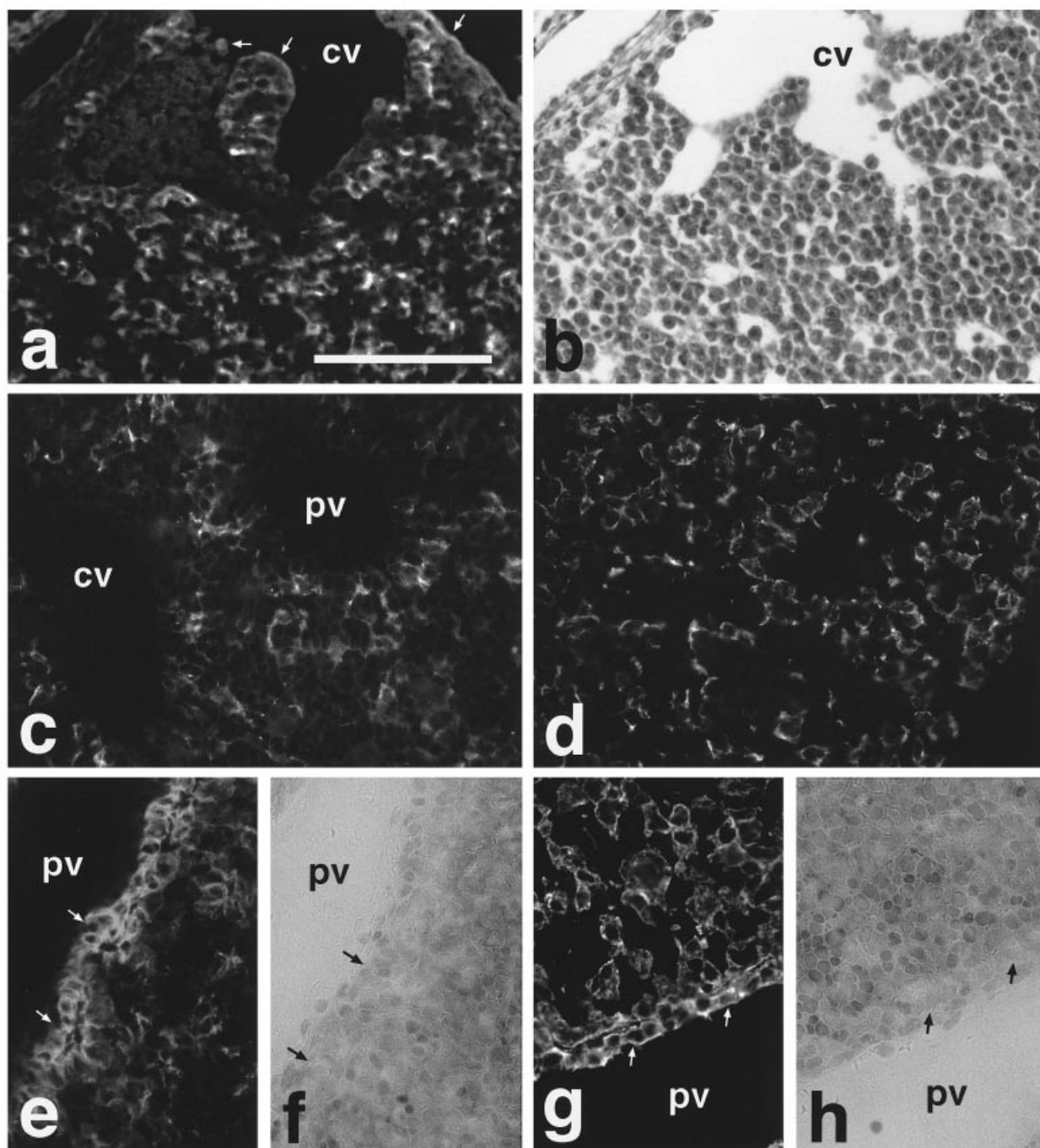


Fig. 6. Expression of albumin (*a*), E-cadherin (*c*, *e*) and cytokeratins (*d*, *g*) in 12.5 d (*a-d*) and 17.5 d (*e-h*) fetal mouse livers. (*b*, *f*, *h*) Haematoxylin-eosin staining of the sections adjacent to *a*, *e* and *g*, respectively. (*a*) The cytoplasm of hepatoblasts is strongly positive for albumin immunostaining. Some endothelial cells, connective tissue cells and blood cells also have positive immunoreactions (arrows). (*e*) E-cadherin expression is visible on the cell membrane of cuboidal hepatoblasts. (*d*) Hepatoblasts contain networks of cytokeratin-positive filaments in their cytoplasm. (*e*, *g*) Biliary epithelial cells and their precursors around portal veins have strong expression of E-cadherin and cytokeratins (arrows). cv, central vein; pv, portal vein. Bar (*a*), 100  $\mu$ m.

#### Primary culture of fetal liver cells

Primary culture of 12.5-d fetal liver cells was carried out to investigate whether hepatoblasts express desmin *in vitro*. After 1 d of primary culture, hepatoblasts formed spherical aggregates by tight adhesion between them (Fig. 8*a*). They were AFP-,

albumin-, cytokeratin- and E-cadherin-positive, but were never positively stained for desmin (Fig. 8*a-d*). Some fibroblastic cells adhered to the aggregates consisting of hepatoblasts. Fibroblastic cells also spread on the slide, and some of the fibroblastic cells contained desmin-positive filaments in their cytoplasm (Fig. 8*c, d*). However, these desmin-positive cells were



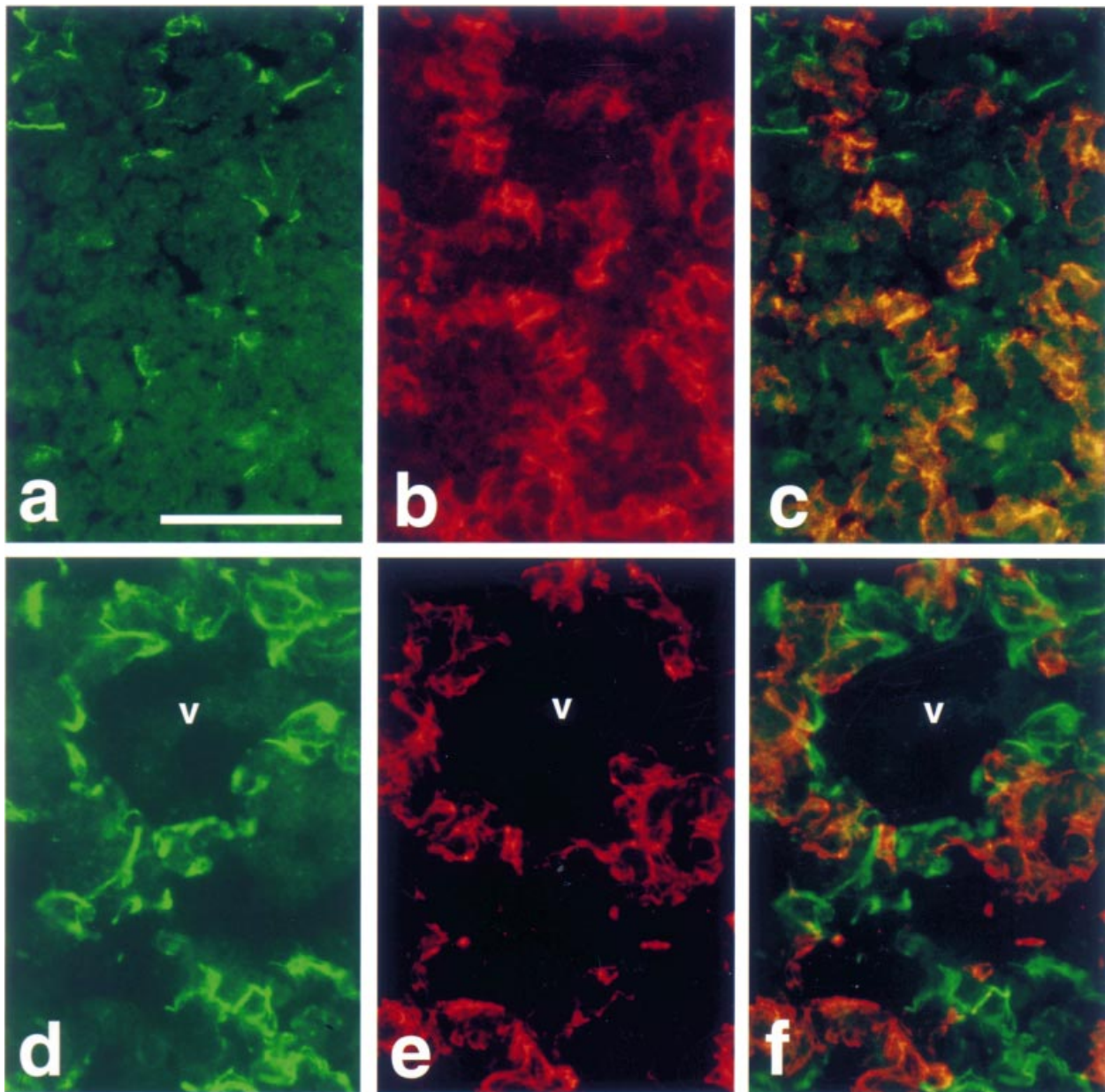


Fig. 7. Double immunofluorescence staining of desmin (*a*) and E-cadherin (*b*), and desmin (*d*) and cytokeratins (*e*) in 11.5-d fetal mouse liver. (*c*, *f*) Double-exposed micrographs of *a* and *b*, and *d* and *e*, respectively. Desmin-positive cells are closely adjacent to E-cadherin- and cytokeratin-positive hepatoblasts, but do not express these hepatoblast markers. Desmin-positive cells also surround blood vessels discontinuously (*d-f*). v, blood vessels. Scale bar (*a*), 50  $\mu$ m.

not stellate, but spindle-shaped or flattened. E-cadherin and cytokeratin immunoreactions were not detected in most of the desmin-positive or negative fibroblastic cells. Some desmin-negative fibroblastic cells were positively stained for cytokeratins, but not for E-cadherin. Haematopoietic cells, which could not be perfectly removed in our isolation protocol, were also observed in this culture (Fig. 8*b*).

After 3 d of the primary culture, spherical aggregates composed of hepatocytes became attached to the slide and started to take on a cobblestone appearance. Hepatocytes forming these aggregates

expressed CPSI (Fig. 8*e*) in addition to AFP, albumin and E-cadherin, but never reacted with antidesmin antibodies (Fig. 8*g*, *h*). The number of hepatocytes increased 1.7-fold between d 1 and 3. Fibroblastic cells, which were desmin-positive or negative, also grew and formed large colonies on d 3, and often surrounded hepatocyte colonies (Fig. 8*f*, *h*). These fibroblastic cells did not express either CPSI or E-cadherin. Desmin-positive cells neither took on a stellate morphology, nor extended desmin-positive cytoplasmic processes to hepatocytes on d 3 (Fig. 8*g*, *h*).



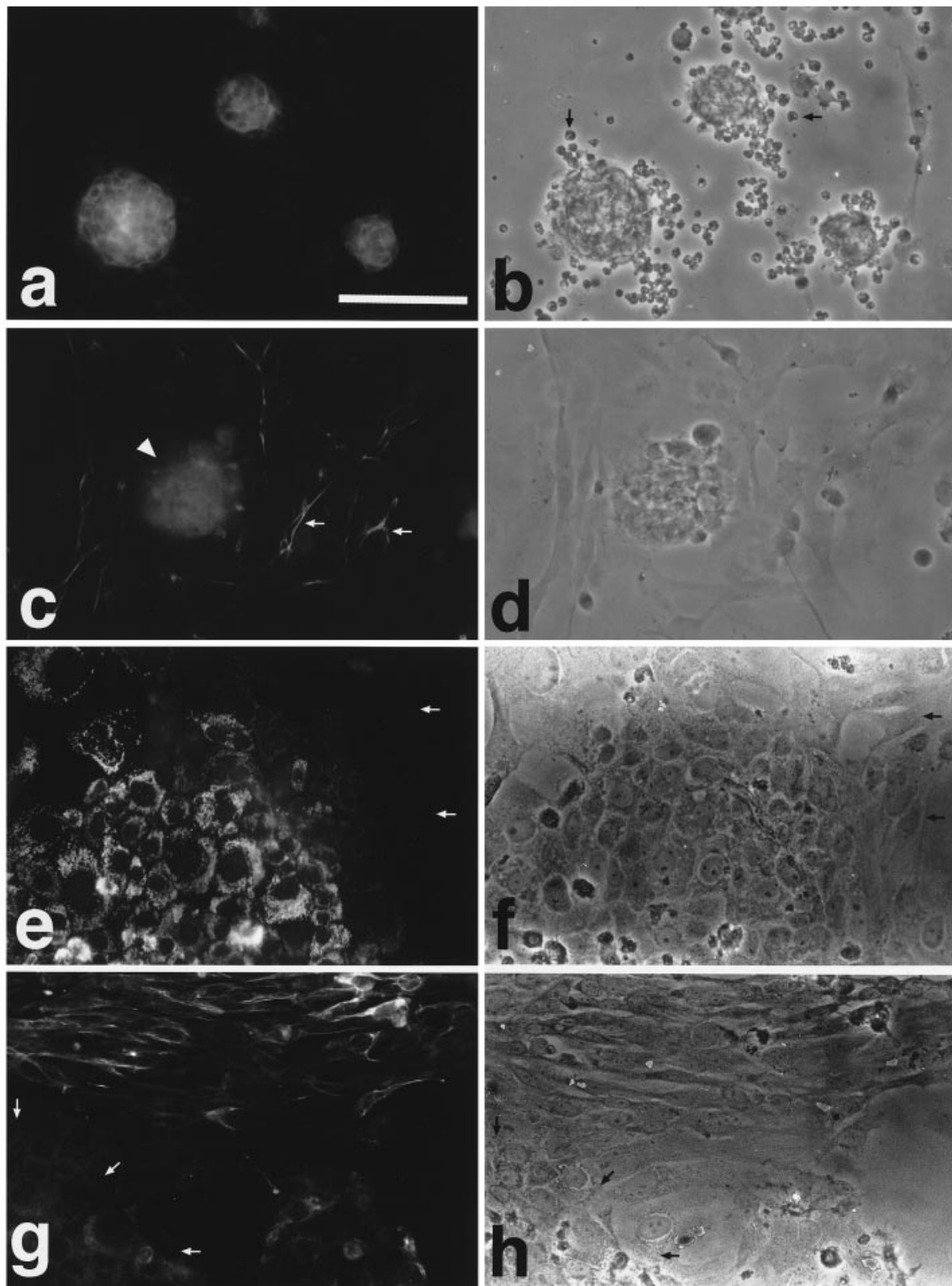


Fig. 8. Immunolocalisation of albumin (*a*), desmin (*c*, *g*) and CPSI (*e*) in 12.5 d fetal mouse liver cells cultured for 1 d (*a–d*) or for 3 d (*e–h*) in vitro. (*b*, *d*, *f*, *h*) Phase-contrast micrographs of the corresponding regions shown in *a*, *c*, *e* and *g*, respectively. (*a*, *b*) Hepatoblasts forming spherical aggregates are albumin-positive. Most single and round cells are haematopoietic cells (arrows). (*c*, *d*) Some fibroblasts spreading on the glass slide are positive for desmin (*c*, arrows). Hepatoblasts of an aggregate do not show positive immunoreaction (*c*, arrowhead). (*e*, *f*) Aggregates consisting of CPSI-positive hepatocytes take on a cobblestone appearance. Fibroblasts surrounding the aggregates are negative for CPSI (arrows). (*g*, *h*) Desmin-positive or negative fibroblasts form large colonies. Arrows indicate desmin-negative hepatocytes forming aggregates. Bar (*a*), 100  $\mu$ m.

## DISCUSSION

Recent studies reported that, in young fetal rat livers, almost all nonhaematopoietic liver cells, including hepatoblasts, transiently coexpressed desmin and cytokeratins, which were used as markers for hepatic stellate cells and hepatoblasts, respectively (Vassy et al. 1993; Kiassov et al. 1995). To verify whether this phenomenon could be observed in the fetal mouse liver, we studied the immunolocalisation of desmin with E-cadherin and cytokeratins at various stages of mouse liver development.

We found desmin immunostaining in morphologically stellate cells of the fetal liver parenchyma after 11.5 d of gestation, which is consistent with the results of previous studies with fetal rat livers (Vassy et al. 1993; Kiassov et al. 1995). In the double immunofluorescence analysis of desmin and cytokeratins, desmin-positive stellate cells were mostly negative for cytokeratins, though a few of them were positive in fetal mouse livers at 11.5 and 12.5 d of gestation, which correspond to 13.5 and 14.5 d of gestation in rat liver development, respectively. However, hepatoblasts and hepatocytes positive for E-cadherin never expressed desmin in fetal mouse livers, including 11.5 and 12.5 d livers. Furthermore, it was very rare for desmin-positive stellate cells to be E-cadherin-positive in fetal mouse livers. In addition, hepatoblasts were morphologically cuboidal, but not stellate. These results clearly indicated that desmin-positive stellate cells were distinct from hepatoblasts in terms of morphology and expression of E-cadherin and desmin. Our culture studies also demonstrated that, when 12.5 d fetal mouse liver cells were cultured, desmin-positive cells were never positive for E-cadherin and cytokeratins, which hepatoblasts and hepatocytes also expressed *in vitro*. Hepatoblasts and hepatocytes *in vitro* never reacted with antidesmin antibodies. Therefore, it is likely that hepatoblasts give rise to hepatocytes, but not to stellate cells in mouse liver development, and that hepatoblasts and stellate cells belong to different cell lineages. We also showed that E-cadherin was expressed in endodermal cells of the 9.5 d liver primordium, but not in its mesenchymal cells. Conversely, positive immunoreaction of desmin was found in most of the mesenchymal cells, but not in the hepatic endodermal cells. These results suggest that hepatoblasts derive from the endodermal cells of the liver primordium, and stellate cells from the septum transversum mesenchyme.

These conclusions do not agree with the idea proposed in previous immunohistochemical studies

(Vassy et al. 1993; Kiassov et al. 1995). The reason for this contradiction can be explained by different specificities of the antibodies against desmin and cytokeratins used in the present study and in previous studies. The positive immunoreaction of our anticytokeratin antibodies to a few young stellate cells possibly stems from their cross-reactivities with other proteins, including vimentin and desmin. Our immunoblotting analysis showed that the antibodies to cytokeratins specifically reacted with 2 major bands corresponding to cytokeratins 8 and 18 in adult mouse liver extract. Although the differences of the animals used may also be a possible cause for the contradiction, our preliminary study indicated that a few stellate cells were positively stained for both desmin and cytokeratins in the 13.5 d fetal rat liver (Nitou et al. unpublished data), which is very similar to the present data for the fetal mouse liver.

The neural crest origin of hepatic stellate cells is assumed, based on the observation that they also express neural cell adhesion molecules, glial fibrillary acidic protein and nestin (Buniatian et al. 1996; Knittel et al. 1996; Nakatani et al. 1996; Neubauer et al. 1996; Niki et al. 1999). These markers are expressed in derivatives of neural crest cells such as astroglial cells. Thus experimental studies on the origin of the stellate cells are required in the future.

Although it is well established that hepatic stellate cells store abundant vitamin A (Blomhoff et al. 1985) and are involved in production of the ECM in mammalian adult liver (Friedman et al. 1985; Milani et al. 1989; Gressner & Bachem, 1995), it still remains to be clarified what roles they play in hepatic development. During fetal development, stellate cells start to accumulate lipid droplets (Matsumoto et al. 1984; Enzan et al. 1997), and can produce ECM components (Baloch et al. 1992; Amenta & Harrison, 1997). Matsumoto et al. (1984) also indicated that the concentration of vitamin A in lipid droplets of stellate cells increased in livers of mouse neonates after commencement of suckling. In the present study, we demonstrated that during mouse liver development, not only geographical changes of desmin-positive stellate cells in the liver parenchyma, but also changes in morphology of their desmin-positive cellular processes occurred. In fetal stages, desmin-positive cellular processes of stellate cells were comparatively thick, but became thinner after birth. The density of desmin-positive stellate cells in the liver parenchyma increased postnatally. These changes may be related to the various functions and maturation of stellate cells.

The present study indicated that, in fetal mouse liver parenchyma, desmin-positive stellate cells were

closely adjacent to other cell types, including endothelial cells of blood vessels, megakaryocytes, haematopoietic cells, hepatoblasts and hepatocytes, implying that stellate cells may control growth, differentiation, or morphogenesis of these cells. It has been demonstrated that matrix metalloproteinase-2 from stellate cells can be activated through interactions with hepatocytes from adult liver (Theret et al. 1997), and that coculture of adult hepatocytes with hepatic stellate cells is beneficial for growth and stability of hepatocytes (Okamoto et al. 1998; Mitaka et al. 1999). In our in vitro culture study showing that hepatoblasts proliferated well and matured to large hepatocytes expressing CPSI, most desmin-positive cells neither adhered to aggregates of hepatocytes nor extended their cellular processes into the aggregates. These results suggest that proliferation and differentiation of hepatoblasts can be partially induced in vitro without their direct contact with desmin-positive cells. Fetal desmin-positive cells might interact with hepatoblasts or hepatocytes via secretion of soluble factors or production of ECM (Baloch et al. 1992; Amenta & Harrison, 1997). In postnatal liver development, all hepatocytes became closely associated with stellate cells. Changes in the morphology of cellular processes containing desmin-positive filaments in stellate cells also occurred after birth. Furthermore, it has been shown that hepatocyte maturation such as expression of asialoglycoprotein receptors and cell adhesion molecules, and liver lobule formation also take place after birth (Petell et al. 1990; Stamatoglou et al. 1992; Thompson et al. 1993; Alexander et al. 1997). These data suggest that hepatic stellate cells may play important roles in these phenomena.

It is also noteworthy that most of the fetal desmin-positive cells became spindle-shaped, rather than typical stellate shape in our cultures. Because culture of adult desmin-positive hepatic stellate cells on type I collagen gel has been shown to be essential for induction of their stellate shape (Imai & Senoo, 1998; Kojima et al. 1998), such culture conditions may also be required for the stellate morphology of fetal-liver desmin-positive cells. The morphology of fetal desmin-positive cells can also be regulated by ECM components in vivo.

In conclusion, hepatoblasts and desmin-positive stellate cells may belong to different cell lineages in fetal mouse liver. Developmental changes in the width of desmin-positive cellular processes in the stellate cells, and in their distribution patterns may be related to their maturation and cell-cell interactions with other cell types, including hepatoblasts, hepatocytes,

endothelial cells, haematopoietic cells and megakaryocytes.

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